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Review

Determination of dissociation constants of polyprotic acids from chromatographic data

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Abstract

The separation and purification of drugs and biological compounds, which are typically weak polyprotic acids, by HPLC is the subject of numerous literature reports. The development of separation methodologies depends on the acid dissociation constants, and the HPLC offers a valuable method for determining these constants, especially when the compounds are poorly soluble in water. This review presents general basic equations and shows how they are used for determining the pK_a's. It also discusses the parameters affecting the pK_a's and the methods of their measurement as presented in a representative number of research papers published in the last 20 years.

Keywords: Reviews; Dissociation constants; Polyprotic acid

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1. Introduction

The usual chromatographic procedures that are used to separate organic acids by liquid chromatography (LC) make use of buffered mobile phases. Under such conditions, the separation depends on several factors of which the dissociation constants of the acid solute play an important role. Thus, to efficiently develop better methods for the separation of organic acids by LC, the dissociation constants of the acid analyte need to be known. Because organic acids tend to be poorly soluble in water, the classical techniques for the determination of dissociation constants are not practical. In the last twenty years liquid chromatography has been used to determine dissociation constants of ionogenic analytes. An added advantage of the LC method is that the sample need not be of high purity. Also nanogram quantities of the analyte can be used in an LC analysis, and this can be an important consideration when the analyte is a biologically important compound available only in small quantities.

Some of the chromatographic techniques that have been used to determine acid dissociation constants are ion chromatography [1], ion-exchange chromatography [2], gas chromatography [3], and even paper chromatography [4]. In more recent years capillary electrophoresis has also been used for this purpose [5,6]. However, reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely used among the separation techniques for determining acid dissociation constants [7].

The calculation of the dissociation constants from chromatographic data requires an understanding of the secondary equilibria that take place in the mobile phase, that is the equilibrium dissociations of the analyte, and how they affect the retention factor. It also requires the establishment of a standard computational procedure which must be based on a general mathematical formulation of the theory of reversed-phase chromatography. Therefore, we will outline the theoretical foundation pertinent to the calculation of the dissociation constants of acidic analytes, mainly, the solvophobic theory of Horváth et al. [8–10]. We will then present a general mathematical formulation which constitutes the ground for obtaining the dissociation constants of acidic analytes.

It was pointed out in the seventies that a stationary phase of hydrophobic *n*-octadecyl groups attached to a silica surface will have a weak and non-selective interactions with solutes, provided residual silanols do not contribute to the overall retention [11]. It has also been shown that log k, where k is the retention factor, is proportional to the log S, where S is the solubility of the solute in water. This is consistent with a hydrophobic interaction between the solute and water. Hermann [reference cited in Ref. [11]] was able to relate the calculated surface area of the water cavity surrounding the hydrocarbon solute to the solubility of the solute. Since the surface area of the cavity is related to the volume and shape of the cavity, it was suggested that a topological molecular-connectivity index may be used to predict relative retention [11]. Topology, however, takes into account the geometry (and hence the volume) of the skeleton of the solute molecule, but it ignores the important effect of the charge density distribution which may play an important role in the interaction of the solute with the solvent or with the stationary phase. Therefore, topology is not an adequate basis for the development of a general theory of polarography, and will not be discussed further in this review.

It has also been recognized by several authors that weak acids, bases, and zwitterionic compounds could be separated by a reversed high-performance liquid chromatography using nonpolar stationary phases. The eluent can be an aqueous solution of ethanol or acetonitrile. Horváth et al. [8,9], and Pietrzyk et al. [12,13], as well as other investigators [14–17], have demonstrated that the chromatographic behavior of the solutes are basically controlled by two major effects, a hydrophobic effect and a reversible ionization of the solute in the mobile phase. An electrically neutral solute species is repelled partially by the solvent. This causes the solute either to be encased in cavities or move towards the interface between the mobile and the stationary phases, where it interacts with the stationary phase and form with it a more or less weak association complex. Such an interaction causes the retention time of the solute to be relatively large. An ionic solute species, on the other hand, is more soluble in the mobile phase and, consequently, has a smaller retention. Therefore, the secondary reversible dissociation of solutes containing ionogenic functions plays an important role in de-

termining the chromatographic behavior of the solute. Such a dissociation is characteristic of the solute. It is determined mainly by the pK_a's or the pK_b's of the solute and the pH of the mobile phase. It is also influenced to a certain extent by the ionic strength of the mobile phase.

The nature of the interaction between the solute and the nonpolar stationary phase, and the kind of the complex thus formed depend on the type of the stationary phase, the composition of the mobile phase, and the structure of the solute. Horváth et al. [9] maintain that when a nonpolar stationary phase is used the physicochemical phenomena underlying the chromatographic process can be interpreted in the light of the solvophobic theory. However, Pietrzyk et al. [12,13] have observed that factors such as charge–transfer interactions, may not be ruled out, especially when the stationary phase contains phenyl groups, as in the case of the Amberlite XAD-2 adsorbent. Heron and Tchapla [18] also observed that in addition to the solvophobic effect, specific interactions, such as $\pi-\pi$ (and possibly $\sigma-\pi$) interactions may take place between the solute and the mobile-phase solvent. Solutes having aromatic ring or a delocalized double-bond system may experience this type of interaction with acetonitrile when used as a modifier in the mobile phase. It was also suggested that amphiprotic solutes may be involved in hydrogen bonding [19]. The solvophobic theory was also criticized for attributing the retention process to the mobile phase 'ignoring contributions from bonded stationary phase'. According to Dorsey and Cooper [20], the solute is partitioned between the mobile phase and the stationary phase, rather than being adsorbed on the stationary phase. The partitioning is defined as full embedding of the solute between the chains of the stationary phase. This partitioning is regulated by the chemical potential difference of the solute between the two phases [20].

Regardless of the nature of interactions involved, however, it is widely accepted that the chromatographic behavior of ionogenic solutes in columns of nonpolar stationary phases is controlled by two main events, the dissociation of the solutes in the mobile phase and the interaction between the solute species and the stationary phase. These events, although interrelated, can be treated theoretically as separate phenomena. In fact, the interaction between the solute and the stationary state affects the limiting retention factors of the solute species, and these factors can be treated as empirical parameters. Whether the solute-stationary phase interaction leads to weak complex formation or to partitioning, there will be an equilibrium between the concentration of the solute in the mobile phase and its concentration in the stationary phase. This equilibrium makes it possible to express the concentration of the solute in the stationary phase in terms of its concentration in the mobile phase. Consequently, it is possible to derive equations describing the retention process in terms of the dissociations taking place in the mobile phase and in terms of some parameters, such as the limiting retention factors, that account for the effect of the stationary phase on the retention of the solute. Based on this, we will generalize the formalism of the solvophobic theory [9] and derive the equations necessary for the calculation of the dissociation constants. We will show that the equations are valid regardless of the nature of the effect of the stationary phase on the retention process.

2. The theory of solvophobic chromatography

Horváth, Melander, and Molnár have developed a theory for the interaction of the solute with nonpolar stationary phases in liquid chromatography [8,9]. The authors had observed that neat aqueous solvents which do not contain organic solvents could be used for the separation of relatively polar biological compounds on octadecylsilica columns. They stipulated that in such cases, the interaction between the solute and the hydrocarbonaceous ligand has to be the sole cause of the solute retention, and that the chromatographic process is governed by a hydrophobic effect where the non polar moiety of the solute interacts closely with the octadecyl chains bonded to the silica phase. Thus, the chromatographic process is treated as a reversible association of the solute with the hydrocarbonaceous ligands of the stationary phase. This proposition is supported by the experimental fact that the retention increases with the hydrocarbonaceous surface area of different classes of solutes such as alkanes, alkanes derivatives, acids, amino acids, and amines. Experiments also revealed that stationary phases of higher carbon

content yielded higher retention under otherwise equivalent conditions.

Sinanoğlu's solvophobic theory [references cited in [8]] is called upon to identify and estimate the magnitude of factors, other than the dissociation in the mobile phase, that may influence the chromatographic process. The authors called this type of reversed liquid chromatography the solvophobic chromatography.

The theory was developed first for unionized solutes [8] and, later on was generalized to include ionizable substances [9]. Since the majority of biological compounds contains ionizable functions such as carboxylic functions and amino groups, we will focus the attention here on the main feature of the theory as it is applied to such compounds.

Equations relating the observed retention factor of monoprotic and diprotic acids to the pH of the mobile phase and the dissociation constants of the acid were derived by Horváth et al. [9]. Similar equations were also obtained by Pietrzyk et al. [12,13], and Foley and May [15,16]. Roses et al. [21] derived equations for monoprotic acid relating the holdup time of the solute to the pH, pK_a of the acid, and the activity coefficient of the anion of the acid. More recently, Jano et al. [22] have generalized the treatment initiated by Horváth and collaborators and obtained a general equation relating the retention factors of polyprotic acids, including amino acids, to the dissociation constants and the pH of the mobile phase. They showed that the equation is valid regardless of the nature of the interactions between the solute species and the stationary phase. The nature of such interaction affects only the limiting retention factors of the individual species which result from the dissociation of the acid. However, the limiting retention factors can be regarded as parameters that can be determined empirically from the experimental measurements of the retention factor of the acid as a function of the pH. The general treatment of Ref. [22] will be adapted here with a slight modification to include the effect of the ionic strength of the mobile phase on the retention factors. This will be accomplished by taking into consideration the activity coefficients of the different species in the solution. Therefore, for the benefit of the reader, basic equations are derived in this report, and examples on their utilization are presented. It will be shown how these equations can be used to determine the retention factors of the individual solute species and the dissociation constants of polyprotic acids directly from the experimental measurements.

We will consider first the dissociation events in the mobile phase and derive basic equations for these events. Then, we will treat the association phenomena between the solute species and the stationary phase following the treatment of Horváth and collaborators. Finally, we will combine the basic equations pertaining to the events in the mobile phase with those related to the associations with the stationary phase, and obtain general formulation for the chromatographic behavior of ionogenic solutes. The characterization of the nature of the variables affecting the dissociation constants of the acid solutes will be discussed.

3. General formulation

3.1. Basic equations

A polyprotic acid, H_nA, ionizes successively in the mobile phase to produce several anions as summarized in Table 1.

Table 1
Dissociation equilibria of polyprotic acid and the equilibrium-constant expressions

$H_nA \rightleftharpoons H_{n-1}A^- + H^+$	$\frac{K_{a,1}}{a(H^+)} = \frac{a(H_{n-1}A^-)_m}{a(H_nA)_m}$
⋮	
$H_{n-(r-1)}A^{(r-1)-} \rightleftharpoons H_{n-r}A^{r-} + H^+$	$\frac{K_{a,r}}{a(H^+)} = \frac{a(H_{n-r}A^{r-})_m}{a(H_{n-(r-1)}A^{(r-1)-})_m}$
⋮	
$HA^{(n-1)-} \rightleftharpoons A^{n-} + H^+$	$\frac{K_{a,n}}{a(H^+)} = \frac{a(A^{n-})_m}{a(HA^{(n-1)-})_m}$

$K_{a,r}$ is the dissociation equilibrium constant of the r^{th} dissociation step. a is the activity of the solute species, and m stands for the mobile phase. Successive multiplications of the first r-equilibrium expressions and the addition of the corresponding equilibrium equations lead to the following general equations:

$$\mathrm{H}_n\mathrm{A} \rightleftharpoons \mathrm{H}_{n-r}\mathrm{A}^{r-} + r\mathrm{H}^+;$$
$$\frac{K(r)}{a^r(\mathrm{H}^+)} = \frac{a(\mathrm{H}_{n-r}\mathrm{A}^{r-})_\mathrm{m}}{a(\mathrm{H}_n\mathrm{A})_\mathrm{m}} \quad (1)$$

where

$$K(r) = K_{a,1} \times K_{a,2} \times \cdots \times K_{a,r} = \prod_{i=1}^{r} K_{a,i} \quad (2)$$

It is clear that the quantity $K(r)$ is the thermodynamic equilibrium dissociation constant of the reaction that leads to the formation of the anion $\mathrm{H}_{n-r}\mathrm{A}^{r-}$ directly (in one step) from the neutral acid (Eq. (1)). It will be shown later that $K(r)$ is related to the type of solvent and the ionic strength of the mobile phase. Eq. (1) allows the calculation of the activity of the anion $\mathrm{H}_{n-r}\mathrm{A}^{r-}$ in terms of the activity of the neutral acid molecule:

$$a(\mathrm{H}_{n-r}\mathrm{A}^{r-})_\mathrm{m} = a(\mathrm{H}_n\mathrm{A})_\mathrm{m} \cdot K(r)/a^r(\mathrm{H}^+) \quad (3)$$

The activity of the acidic proton H^+ is related to the pH of the solution and can be expressed as:

$$a(\mathrm{H}^+) = 10^{-\mathrm{pH}} = e^{-x};\ x = (\ln 10) \cdot \mathrm{pH} \quad (4)$$

and Eq. (3) is written as:

$$a(\mathrm{H}_{n-r}\mathrm{A}^{r-})_\mathrm{m} = a(\mathrm{H}_n\mathrm{A})_\mathrm{m} \cdot K(r) \cdot e^{rx} \quad (5)$$

The activity of a species is considered in general equal to the concentration of the species multiplied by an activity coefficient. Therefore Eq. (5) can be written in the following form:

$$\gamma(r)[\mathrm{H}_{n-r}\mathrm{A}^{r-}]_\mathrm{m} = \gamma(0)[\mathrm{H}_n\mathrm{A}]_\mathrm{m} \cdot K(r) \cdot e^{rx}$$

Or:

$$[\mathrm{H}_{n-r}\mathrm{A}^{r-}]_\mathrm{m} = [\mathrm{H}_n\mathrm{A}]_\mathrm{m} \cdot f(r) \cdot K(r) \cdot e^{rx} \quad (6)$$

$$f(r) = \gamma(0)/\gamma(r) \quad (7)$$

$\gamma(0)$ and $\gamma(r)$ are the activity coefficients of the neutral acid and the anion $\mathrm{H}_{n-r}\mathrm{A}^{r-}$ respectively. Summation of Eq. (6) over all the species, $r = 0, 1, \ldots, n$, yields:

$$\sum_{r=0}^{n} [\mathrm{H}_{n-r}\mathrm{A}^{r-}]_\mathrm{m} = [\mathrm{H}_n\mathrm{A}]_\mathrm{m} \sum_{r=0}^{n} f(r) \cdot K(r) e^{rx} \quad (8)$$

By dividing Eq. (6) by Eq. (8), one obtains

$$X(r) = \frac{[\mathrm{H}_{n-r}\mathrm{A}^{-}]_\mathrm{m}}{\sum_{r=0}^{n} [\mathrm{H}_{n-r}\mathrm{A}^{r-}]_\mathrm{m}} = \frac{f(r) \cdot K(r) e^{rx}}{\sum_{r=0}^{n} f(r) K(r) e^{rx}} \quad (9)$$

$X(r)$ is the mole fraction (i.e. relative concentration) of the species having a net charge equal r ($r = 0, 1, \ldots, n$). It is clear that the sum of the mole fractions is equal to 1. Eq. (9) is written more conveniently as

$$X(r) = \frac{f(r)K(r)e^{rx}}{1 + \sum_{r=1}^{n} f(r)K(r)e^{rx}} \quad (10)$$

This is because $f(0) = \gamma(0)/\gamma(0) = 1$, and $K(0) = 1$ by definition.

Eq. (10) is basic. It shows how the mole fraction varies with the pH of the mobile phase. This equation can be used to predict which species will be the major contributor to the measured retention factor of the acid as will be explained later.

Now we consider the association between the solute and the stationary phase. In general, an entity $\mathrm{H}_{n-r}\mathrm{A}^{r-}$ (where $r = 0$ corresponds to the neutral acid molecule and $r = 1, 2, \ldots, n$ correspond to the anions that result from the dissociation of the acid) is assumed to form an association complex, $\mathrm{LH}_{n-r}\mathrm{A}^{r-}$, with the ligand L bonded to the stationary phase

$$\mathrm{H}_{n-r}\mathrm{A}^{r-} + \mathrm{L} \rightleftharpoons \mathrm{LH}_{n-r}\mathrm{A}^{r-};$$
$$K_{\mathrm{L},r} = \frac{a(\mathrm{LH}_{n-r}\mathrm{A}^{r-})_\mathrm{s}}{a(\mathrm{H}_{n-r}\mathrm{A}^{r-})_\mathrm{m} \cdot a(\mathrm{L})_\mathrm{s}} \quad (11)$$

$K_{\mathrm{L},r}$ is the association equilibrium constant. The parameters s and m stand for the stationary and mobile phases respectively. The expression of the equilibrium constant can be written in the following form:

$$a(\mathrm{LH}_{n-r}\mathrm{A}^{r-})_\mathrm{s} = K_{\mathrm{L},r}\, a(\mathrm{H}_{n-r}\mathrm{A}^{r-})_\mathrm{m} \cdot a(\mathrm{L})_\mathrm{s} \quad (12)$$

The observable (i.e. measurable) retention factor, k, of the acid is by definition given by

$$k = \phi \frac{\sum_{r=0} [LH_{n-r}A^{r-}]_s}{\sum_{r=0} [H_{n-r}A^{r-}]_m} \quad (13)$$

ϕ is the volume ratio of the stationary and mobile phases. The ratio ϕ is usually maintained constant in a given column. Similarly, the retention factor k_r of $H_{n-r}A^{r-}$ is expressed as

$$k_r = \phi \frac{[LH_{n-r}A^{r-}]_s}{[H_{n-r}A^{r-}]_m} \quad (14)$$

Eq. (14) permits the calculation of the concentration of the complex, $[LH_{n-r}A^{r-}]_s$, in terms of the concentration of the corresponding anion in the mobile phase

$$[LH_{n-r}A^{r-}]_s = \frac{k_r}{\phi} [H_{n-r}A^{r-}]_m \quad (15)$$

Summation of Eq. (15) over all the species, $r=0$, 1,..., n, yields

$$\sum_{r=0}^{n} [LH_{n-r}A^{r-}]_s = \frac{1}{\phi} \sum_{r=0}^{n} k_r [H_{n-r}A^{r-}]_m \quad (16)$$

If one multiplies Eq. (6) by k_r and sum over r, and then combines the resulting equation with Eq. (16) one obtains the following relation

$$\sum_{r=0}^{n} [LH_{n-r}A^{r-}]_s = \frac{1}{\phi} [H_nA]_m \sum_{r=0}^{n} k_r f(r)K(r)e^{rx} \quad (17)$$

The substitution of Eqs. (8) and (17) into Eq. (13) leads to the following relation for the retention factor of the polyprotic acid

$$k = \frac{\sum_{r=0}^{n} k_r \cdot f(r)K(r)e^{rx}}{\sum_{r=0} f(r) \cdot K(r)e^{rx}}$$

This equation is written more conveniently as

$$k = \frac{k_0 + \sum_{r=1}^{n} k_r \cdot f(r)K(r)e^{rx}}{1 + \sum_{r=1}^{n} f(r) \cdot K(r)e^{rx}} \quad (18)$$

This is the most general equation for the retention factor of polyprotic acids. In case the activity-coefficient ratios are equal unity, Eq. (18) reduces to Eq. (12) of Ref. [22]. Equations for monoprotic and diprotic acids can be obtained from Eq. (18) by putting $n=1$ and 2 respectively.

It is important to note here that even though the solute–ligand association equilibrium constants do not appear explicitly in Eq. (18), these constants influence the values of the retention factors indirectly as can be seen from Eqs. (12) and (13).

3.2. The case of amino acids

It can be shown that Eq. (18) also applies to amino acids provided that a proper matching between the limiting retention factors and the acid species is specified. The general molecular formula of an amino acid may be written as $(H_2N)_\mu R_1R_2(COOH)_\nu$. Such a compound exists as $(H_3^+N)_\mu R_1R_2(COOH)_\nu$ in low (acidic) pH values, and as $(H_2N)_\mu R_1R_2(COO^-)_\nu$ at high (basic) pH values. When the pH of the solution is gradually increased from low to high values, the acid undergoes successive dissociations where protons dissociate first from the carboxylic groups, and then from the protonated amino groups NH_3^+. In such events the correspondence between the retention factors of Eq. (18) and the different species is as follows. Denoting the extreme cases of the acid by $H_\mu^+ABH_\nu$ and $AB^{\nu-}$, then retention factors are in the following order:

$$k_0 = k(H_\mu^\mu ABH_\nu)$$

$$k_1 = k(H_\mu^+ ABH_{\nu-1}^-)$$

$$k_\nu = k(H_\mu^+ AB^{\nu-})$$

$$\vdots$$

$$k_{\mu+\nu} = k(AB^{\nu-})$$

where $\mu+\nu=n$. For more details see Ref. [22]. It is obvious that this general treatment includes, as a special case, the zwitter ions ($\mu=\nu=1$).

3.3. Probabilistic interpretation of Eq. (18)

It was recognized that the expression of the observable retention factor of the ionogenic solute, as given by the general Eq. (18), has probabilistic characteristics [22]. In view of Eq. (9), Eq. (18) can be written as a weighted average of the limiting retention factors k_r.

$$k = k_0 X(0) + \sum_{r=1}^{n} k_r \cdot X(r) \tag{19}$$

The expression of the mole fractions, Eq. (9), has the properties of a normalized probability distribution function. This implies that the term $X(r)$ represents the probability that the measured retention factor of the analyte is equal the retention factor k_r, and Eq. (19) implies that the measured retention factor is the probability weighted average of the limiting retention factors of the species existing in the mobile phase. This interpretation of Eq. (18) (or Eq. (19)) makes it possible to predict which species is the main contributor to the observed retention of the analyte at a given pH of the mobile phase. The species that has the maximum presence in the mobile phase is the main contributor to the measured k. The identification of the dominant species at a given pH can be made as follows. Setting the derivative of $X(r)$ (Eq. (9)) with respect to $x(x = \mathrm{pH} \cdot \ln 10)$ equal to zero, and solving the resulting equation for r yields

$$r = \frac{\sum_{t=1}^{n} t \cdot f(t)K(t) \cdot e^{tx}}{1 + \sum_{t=1}^{n} f(t)K(t) \cdot e^{tx}} \tag{20}$$

The variable r, which identifies the solute species, takes only integer values, 1, 2,.., n. For each one of these values there is a corresponding pH that satisfies Eq. (20). To find the pH that corresponds to a certain value of r (i.e. to maximum presence of an anion) the function r can be plotted as a continuous function of pH, and the pH that corresponds to the integer value of r is then determined. An example is provided below.

3.4. Calculation of the dissociation constants

Eq. (18) can be used for calculating the dissociation constants of the polyprotic acid and the limiting retention factors from the chromatographic data by a nonlinear iterative least square fitting procedure [22]. The dissociation constants, $K(r)$, in all basic equations, are multiplied by the activity coefficient ratios, $f(r)$. A product $K(r) \cdot f(r)$ can be considered as an effective dissociation constant of the direct formation of the anion $H_{n-r}A^{r-}$ from the neutral acid (see Eqs. (1) and (2)).

$$K_e(r) = f(r) \cdot K(r) \tag{21}$$

The effective dissociation constants depend on the nature of the solvent and the ionic strength of the mobile phase. Therefore, they can be invariant only under constant ionic strength conditions for a given solvent composition. Such conditions may be realized by adding an inert salt to the solution of the analyte. To control the pH of the mobile phase, buffer systems have to be designed in such a way as to maintain constant ionic strength at different pH's (see [16]). In the following sections we will assume that the ionic strength remains constant during the variation of the pH of the mobile phase. Under these circumstances, the effective dissociation constants, $K_e(r)$, and the limiting retention factors, k_r, may be determined from the chromatographic data by a computational procedure described briefly in Ref. [22] and in more details in Ref. [23] (in connection with calculating the activity coefficients of individual ions from titration data). Once the $K_e(r)$ are determined, the apparent dissociation constants of the acid can be calculated from the $K_e(r)$. This, however, requires defining the apparent dissociation constants of the acid in a manner consistent with the theory outlined above. The apparent dissociation constants of a polyprotic acids are defined as shown in Table 2. In this table, $K_{ap,i}$ represents the apparent dissociation constant of the i^{th} dissociation step of the acid (Table 1), while $K_{a,i}$ is the thermodynamic constant of the same dissociation step. The apparent constant $K_{ap,i}$ is defined as $K_{ap,i} = K_{a,i}(\gamma_{i-1}/\gamma_i)$. Successive multiplications of the apparent dissociation constants, as shown in the second column of Table 2, reveals the general relation:

$$K_e(r) = K_{ap,1} \cdot K_{ap,2} \cdot \cdots K_{ap,r} = \prod_{i=1}^{r} K_{ap,i} \tag{22}$$

Therefore, once the effective dissociation constants, $K_e(r)$, are calculated from the chromatographic mea-

Table 2
The apparent dissociation constants of a polyprotic acid and their relation to the effective dissociation constants

Apparent constant	Successive products
$K_{ap,1} = K_{a,1} \cdot \frac{\gamma_0}{\gamma_1}$	$K_{ap,1} = K_{a,1} \cdot f(1) = K_e(1)$
$K_{ap,2} = K_{a,2} \cdot \frac{\gamma_1}{\gamma_2}$	$K_{ap,1} \cdot K_{ap,2} = K_{a,1} \cdot K_{a,2} \cdot \frac{\gamma_0}{\gamma_1} \times \frac{\gamma_1}{\gamma_2} = K_e(2)$
⋮	
$K_{ap,r} = K_{a,r} \cdot \frac{\gamma_{r-1}}{\gamma_r}$	$K_{ap,1} \cdot K_{ap,2} \cdots K_{ap,r} = K_{a,1} \cdot K_{a,2} \cdots K_{a,r} \cdot \frac{\gamma_0}{\gamma_r} = K_e(r)$
⋮	
$K_{ap,n} = K_{a,n} \cdot \frac{\gamma_{n-1}}{\gamma_n}$	$K_{ap,1} \cdot K_{ap,2} \cdots K_{ap,n} = K_{a,1} \cdot K_{a,2} \cdots K_{a,n} \cdot \frac{\gamma_0}{\gamma_n} = K_e(n)$

surements by a nonlinear least square fitting procedure, the apparent dissociation constants, $K_{ap,i}$, are calculated from Eq. (22).

To obtain the thermodynamic dissociation constants, $K(r)$, of the acid, one has to proceed as follows. The parameters k_r and $K_e(r)$ can be determined at various ionic strengths. Then, the effective $K_e(r)$ are extrapolated to zero ionic strength to obtain the thermodynamic dissociation constants $K(r)$. Eq. (2) is then used to calculate the thermodynamic constants $K_{a,i}$. This is because the effective $K_e(r)$ and thermodynamic $K(r)$ constants become practically identical at zero ionic strength (i.e. infinite dilution of the solutions). This can be seen as follows. The thermodynamic $K(r)$ (see Eq. (1)) can be written as:

$$K(r) = a^r(H^+)\{\gamma(H_{n-r}A^{r-})/\gamma(H_nA)\} \cdot \{[H_{n-r}A^{r-}]/[H_nA]\}$$

$$= a^r(H^+) \cdot (1/f(r)) \cdot \{[H_{n-r}A^{r-}]/[H_nA]\} \quad (23)$$

from which we obtain:

$$f(r) \cdot K(r) = K_e(r) = a^r(H^+) \cdot [H_{n-r}A^{r-}]/[H_nA] \quad (24)$$

At infinite dilution (zero ionic strength) the activity coefficients become equal unity ($f(r) = 1$), and the effective dissociation constant $K_e(r)$ becomes equal to the thermodynamic $K(r)$.

For all practical purposes however, the determination of the effective dissociation constants at constant ionic strength is sufficient for analyzing the chromatographic data, determining optimum separation conditions, and making predictions. For reasons that will be apparent later, the effective dissociation constants have to be determined from the chromatographic data rather than from separate titration measurements using water as a solvent.

3.5. The effect of the solvent and ionic strength

It has been recognized that the dissociation constants of an acid depend on the nature of the solvent and on the ionic strength of the acid solution [21,24–28]. This can be seen from the following theoretical considerations. The dissociation constant K(r) of the dissociation reaction of Eq. (1) can be written as

$$K(r) = \Gamma(r) \cdot K^c(r) \quad (25)$$

where

$$\Gamma(r) = \gamma^r(H^+) \cdot \gamma(H_{n-r}A^{r-})/\gamma(H_nA) \quad (26)$$

$$K^c(r) = [H^+]^r[H_{n-r}A^{r-}]/[H_nA] \quad (27)$$

γ is the activity coefficient, and $K^c(r)$ the equilibrium constant expressed in terms of the concentrations of the species involved in the dissociation reaction. The thermodynamic dissociation constant $K(r)$ is related to the free energy change of dissociation $\Delta G_{d,l}(r)$:

$$-\ln K(r) = \Delta G_{d,l}(r)/RT \quad (28)$$

The dissociation in the liquid phase can be thought of as happening in two steps, dissociation in the gas phase followed by the introduction of the species at equilibrium into the solvent. Therefore, the free

energy change of the dissociation reaction in liquid phase can be expressed in terms of the free energy change of dissociation in the gas phase, $\Delta G_{d,g}$, and the free energy change of solvation

$$\Delta G_{d,l}(r) = \Delta G_{d,g}(r) + r\Delta G_{sol}(H^+) + \Delta G_{sol}(H_{n-r}A^{r-}) - \Delta G_{sol}(H_nA)$$

$$= \Delta G_{d,g}(r) + \Delta\Delta G_{sol} \quad (29)$$

where the solvation free-energy changes are combined in the term $\Delta\Delta G_{sol}$.Combining Eqs. (25) and (28), and Eq. (29) leads to:

$$-\ln \Gamma(r) - \ln K^c(r) = \Delta G_{d,g}(r)/RT + (\Delta\Delta G_{sol})_{\epsilon,I}/RT \quad (30)$$

The subscripts ϵ and I are added to the solvation energy term to indicate that this term depends on the nature of the solvent as identified by the dielectric constant ϵ, and the ionic strength I of the solution. As the ionic strength approaches zero (infinite dilution) the activity coefficients approach unity and Eq. (30), at the limit, becomes

$$-\ln K^c(r) = \Delta G_{d,g}(r)/RT + (\Delta\Delta G_{sol})_{\epsilon,I=0}/RT \quad (31)$$

This equation shows that $K^c(r)$ depends on the nature of the solvent, and in general, it will change from solvent to solvent even at zero ionic strength. Subtraction of Eq. (31) from Eq. (30) yields

$$-\ln \Gamma(r) = (1/RT)[(\Delta\Delta G_{sol})_{\epsilon,I} - (\Delta\Delta G_{sol})_{\epsilon,I=0}] \quad (32)$$

This is an exact thermodynamic equation. It implies that the activity coefficients in a given solution are determined by the solvation energies of the solute species in the pure solvent ($I=0$) and in the solution having an ionic strength I.

Experimental findings confirm the above conclusions [21,24–27]. For example, Hardcastle et al. [27] found that the pK_a's of leukotrienes depended on the volume-composition of the water–organic modifier solvents at constant ionic strength. Bosch et al. [24] derived semiempirical equations relating the dissociation constants of an acid to the composition of the solvent and the ionic strength of the solution. The empirical parameters required for these equations are tabulated for methanol–water solvent [24].

3.6. Measuring the pH

It remains now to look at the question of how the pH of the mobile phase should be measured. It is customary to use mixed solvents in the reversed-phase chromatography, and to regulate the pH by using standard buffers. However, the pH of a buffer is usually determined on the basis of the pK_a of the acid of the buffer, and the pK_a is, in turn, determined in aqueous solution. But when the buffer is added to the mobile-phase mixed solvent, the pK_a will no longer remain the same, and consequently, the pH will change. This problem was recognized and discussed in the literature [21]. Using a semiempirical equation for calculating the pK_a of acids in methanol–water solvent, Bosch et al. [24] found that the pK_a of some acids used in buffers may differ by about one pK_a unit from the pK_a in a water solution. As a result, the pH of the buffer may change significantly upon mixing with the chromatographic mobile solvent.

Eqs. (28)–(32) can be used, in principle, to calculate the dissociation constant of the buffer-acid and the pH of the mobile phase. However, the calculation of the solvation energies is unfortunately, not easy. Debye–Hückel Equation is also not suitable for calculating the activity coefficients in mixed solvents. The answer at present to this problem is either to develop a semiempirical model for calculating the pH as a function of the composition of the solvent and its ionic strength [21,24], or, as suggested by Uhrová et al. [6], to use a scale of pH based on standardizing a set of buffers in the solvent mixture used in chromatographical analysis. The empirical models require determining and tabling several empirical parameters for every type of solvent combination. The use of a standardized pH scale, on the other hand, is more practical and has the advantage of not having to deal with the difference between the thermodynamic pH and the measured pH. The thermodynamic pH is defined as $pH = -\log a(H^+)$, and the measured pH may not coincide with it. The measured pH is conventionally put equal to $-\log a(H^+)$, but this is only an approximation (for example see [29]. Therefore, the dissociation constants calculated from the chromatographic data are meaningful only with reference to the chosen pH-scale, whether it is a semiempirical or

a standardized-buffer-scale, including the pH-measuring device (type of the electrodes). This is true in the measurement of all physical quantities that depend on the pH of the medium.

It can be concluded that, since the dissociation constants depend on the characteristics of the solvent and the ionic strength of the mobile phase, the determination of these constants directly from the chromatographic data should yield dissociation-constant values relevant to the prevailing conditions of the chromatographic process. These values can be used with the basic equations developed above to obtain important information about the secondary events in the reversed-phase liquid chromatography and to make certain predictions.

3.7. Examples

The methodology for obtaining information from the chromatographic data is as follows. The data from the measured retention factor of the acidic analyte as a function of the pH of the mobile phase are fitted to Eq. (18) by a nonlinear least square fitting procedure [22,23]. This allows the calculation of the effective dissociation constants, $K_e(r)=f(r)\cdot K(r)$, and the limiting retention factors, k_r, of the different acid species in the mobile phase. The effective dissociation constants thus calculated are then used to calculate the apparent dissociation constants, K_{ap}'s, using Eq. (22). As an example, Table 3 contains the apparent pK_{ap}'s of leukotriene B4, leukotriene E4, and N-acetylleukotriene E4 obtained from the chromatographic data as described by Hardcastle et al. [28].

The calculated effective constants $K_e(r)$ are also used with Eq. (9) to follow the evolution of the mole fractions of the acid species during the variation of the pH of the mobile phase.

Fig. 1a shows the variation of the retention factor of leukotriene E4 (LTE4) versus the pH in a 60% (v/v) acetonitrile–water solvent. The marked points are measured, and the solid line passing through these points is calculated from Eq. (18). The variations of the mole fractions, Eq. (9), are also plotted on the same figure. It is clear that in a strong acid solution where pH$<$2, the retention factor is relatively high. In this range of the pH, the amine group is protonated, NH_3^+. As the pH increases, the first dissociation step, involving a carboxylic group, takes place and yields a zwitterionic type species. The retention of this species is evidently lower than that of the undissociated LTE4. In the second dissociation step a proton is dissociated from the second carboxylic group yielding a more polar ion having the lowest retention factor. In basic solution, where the pH is above 9, the completely dissociated species is formed, and the retention increases appreciably. The increase of retention is probably an indication of a steric effect that may block the carboxylic group from being solvated.

The retention of the acid is affected by the composition of the mobile phase. Fig. 1b shows the variation of the retention factor of LTE4 in 50% organic modifier. The retentions of the acid species in this case are, in general, higher than in 60% modifier solvent. Fig. 1b reveals similar trend of behavior as in Fig. 1a. However, there is a shift of the order of about 0.5 pH unit in the pH values that correspond to the maximum mole fractions of the intermediate species. This is accompanied by a change of the apparent dissociation constants of the acid and the retention factors [28].

As another example, Fig. 2b shows the variations

Table 3
Calculated pK_a values for the leukotrienes at different percentages of mobile phase organic modifier (Reproduced from Ref. [28] with permission of Elsevier Science B.V.)

% Organic	LTB_4	LTE_4			*N*-Acetyl-LTE_4	
Modifier	pK_{a1}	K_{a1}	pK_{a2}	pK_{a3}	pK_{a1}	pK_{a2}
45	5.92	2.60	5.45	9.66	2.98	5.45
50	6.04	2.82	5.20	9.42	3.04	5.47
55	6.13	3.09	5.74	9.44	3.08	5.60
60	6.29	2.93	5.96	9.44	3.13	5.75

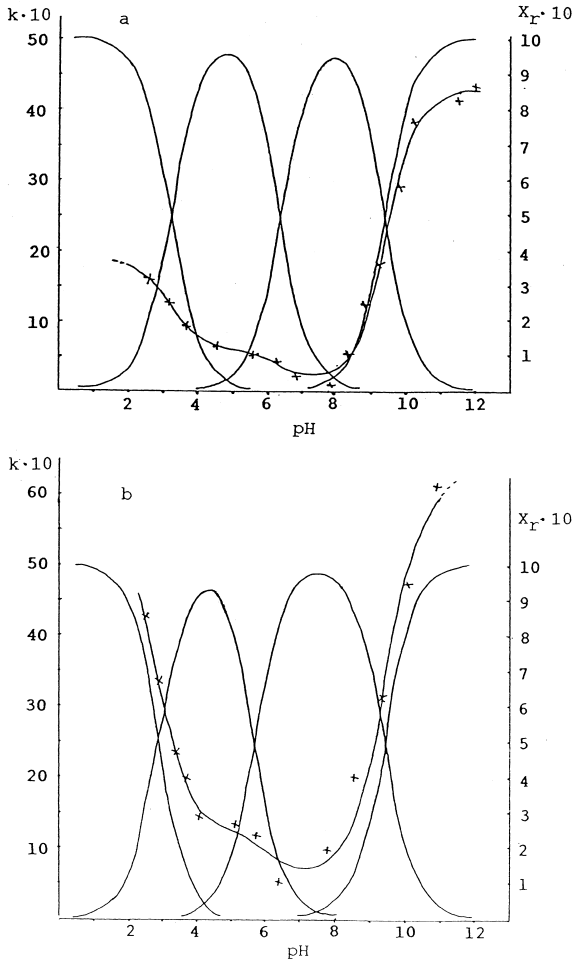

Fig. 1. (a) Variations of the retention factor and mole fractions of leukotriene E_4 (LTE_4) and its dissociated species, as functions of the pH in 60% (v/v) organic modifier (see Ref. [28] for details); (b) Variations of the retention factor and mole fractions of LTE_4 and its dissociated species in 50% (v/v) organic modifier.

of the retention factor and the mole fractions of DL-tyrosine in water in a column of Amberlite XAD-7. Experimental data are obtained from Ref. [14] and processed by our computer program [22]. Tyrosine is an amino acid with a side phenolic group and treated as a triprotic acid. At high pH, the acid is completely dissociated and apparently has a low retention, indicating a relatively high solubility in the mobile phase (buffered water solution). Fig. 2a depicts the variation of r as a function of the pH of the mobile phase (Eq. (20)). Fig. 2a Fig. 2b are plotted on the

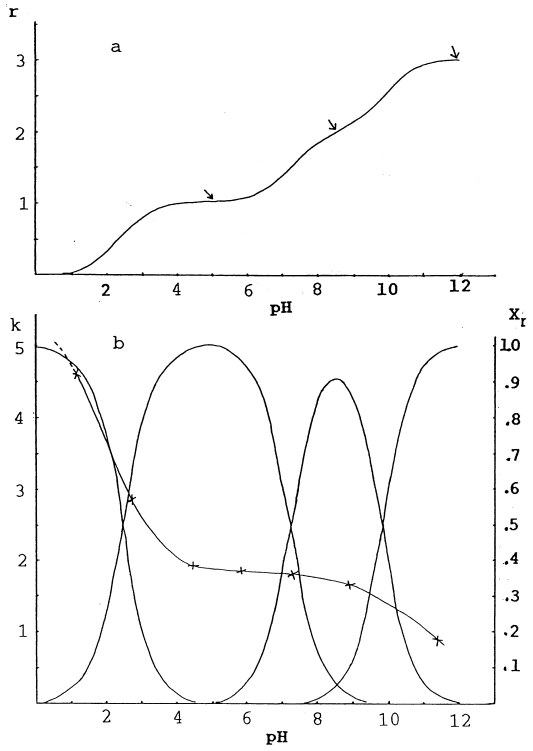

Fig. 2. (a) Variation of *r* (Eq. (20)) with the pH of the mobile phase for DL-tyrosine; (b) Variations of the retention factor and mole fractions of DL-tyrosine and its dissociated species. Experimental data are from Ref. [14].

same pH scale. The *r*-values of 1, 2, and 3 correspond to the maximum-mole- fraction pH's. This can be seen from comparing Figs. 2a and 2b.

3.8. Limitations

Precaution has to be exercised when calculating the dissociation constants of polyprotic acids by least square fitting procedures. Our experience shows that when a polyprotic acid ($n>2$) has a very small dissociation constant corresponding to the last dissociation step, the accuracy of the calculated value of this constant is questionable. One reason for this, is the fact that the measured retention factor is insensitive to variations of very small dissociation constants, especially when the dissociation step takes place in basic solution (pH>8). In such cases, the uniqueness of the dissociation constants' values is not guaranteed. To put it in a mathematical context,

the non approximate fitting procedure requires the calculation of the inverse of a square matrix (for details see Ref. [23]). When the determinant of the matrix is very small (or very large) the accuracy of the calculation is usually compromised. If the determinant is practically zero, then there is more than one solution to the problem. Such situation may be encountered in case of a weak polyprotic acid ($n>2$). This is usually an indication that the measured retention factor is practically independent of the last dissociation constant(s) which is (or are) very small. One way of dealing with this problem is to treat the polyprotic acid as a diprotic acid, and to use data obtained in a limited range of the pH (pH<8) to calculate the first two dissociation constants of the acid. Once these are determined, attempt may be made to determine the remaining dissociation constant(s) by extending the fitting procedure over measurements made in a wider range of pH.

In any case, to insure uniqueness of calculated values, one has to use a sufficiently large number of measured data points (about 6n points) and to make sure that the calculated limiting retention factors and dissociation constants are independent of the initial input values (required for nonlinear iterative fitting procedures). The choice of the initial limiting retention factors may be aided by first plotting the measured retention factor versus the pH. The areas of slow variations on the curve usually correspond to the limiting retention factors of the anions. In acidic solution, where the acid is practically undissociated (pH<3) the measured retention factor is of the order of the limiting factor k_0. In the region of high pH, when the acid is completely dissociated, the measured k is practically equal to the limiting factor, k_n, of the anion A^{n-}. Therefore, these values may be used as input values for the iterative computational process.

3.9. Relative strength of the stationary phase–solute complex

The calculated limiting retention factors may be used to estimate the relative strength of the association complexes between the acid species and the stationary phase. If we consider the activity of the complex $LH_{n-r}A^{r-}$ formed between $H_{n-r}A^{r-}$ and the ligand L, proportional to its concentration in the stationary phase:

$$a(LH_{n-r}A^{r-}) = \gamma_{L,r}[LH_{n-r}A^{r-}] \quad (33)$$

where $\gamma_{L,r}$ is an activity coefficient, then the following relation can be readily obtained from Eqs. (12) and (14), and Eq. (33)

$$K_{L,r}/K_{L,0} = [f(r)/f(L,r)] \cdot (k_r/k_0) \quad (34)$$

$$f(L, r) = \gamma_{L,0}/\gamma_{L,r}$$

$f(r)$ is defined by Eq. (7), and $f(L,r)$ is the ratio of the activity coefficients of the complexes LH_nA and $LH_{n-r}A^{r-}$. The ratio of the association equilibrium constants, $K_{L,r}/K_{L,0}$, is a measure of the strength of the complex $LH_{n-r}A^{r-}$ relative to the strength of the complex LH_nA between the undissociated acid and the ligand. Eq. (34) shows that the relative strength is proportional to the ratio of the limiting retention factors.

Since the association constants are related to the free energy changes of association, Eq. (34) can be used to derive relations between the retention factors and the energetics of the chromatographic association processes. The following equation can be readily obtained from Eq. (34),

$$\ln(k_r/k_0) = \ln[f(L, r)/f(r)] + [\Delta G_{L,0} - \Delta G_{L,r}]/RT \quad (35)$$

where $\Delta G_{L,0}$ and $\Delta G_{L,r}$ are the free energy change of association between the stationary phase and the neutral acid, and the anion r respectively. The study of the variation of $\ln(k_r/k_0)$ with $1/T$ should provide information about the relative strength of associations, as measured by $\Delta G_{L,0} - \Delta G_{L,r}$ which is proportional to the slope of the curve of Eq. (35).

The solvophobic theory [8,9] has attempted to calculate the free energy change of association using Sinanoglu theory combined with other solvent–solute and solute–solute interaction models [8,9]. The authors of the theory derived equations relating the retention factors to a set of variables, some of which may be determined empirically. These relations are approximate due to several simplifying approxima-

tions and assumptions made by the authors. The theory agrees well with the experimental findings for monoprotic acids, but in the case of polyprotic and amino acids the agreement with experiments is less than satisfactory [9]. In addition to the many approximations, the theory ignores specific interactions, such as hydrogen bonding and $\pi-\pi$ interactions, that may take place between the solute and solvent, and between the solute and a stationary phase containing phenyl groups [12,13,17–19]. The solvophobic theory treatment of the energetics of the reversed-phase chromatography may be described at present as exploratory.

4. Measurement of experimental parameters

To be able to use bonded-phase liquid chromatography efficiently for separations and to prepare columns of high stability and reproducibility, the retention mechanism needs to be understood. Dorsey and Cooper [20] state 'a complete understanding of retention will allow researchers to use the chromatographic column to measure physical parameters that are otherwise difficult to obtain.' The solvophobic theory attributes the retention to a solvophobic interaction and weak-complex formation between the bonded ligand and the solute species. This theory as mentioned earlier, ignores other types of interactions that may play a role in the retention mechanism. According to Dorsey and Cooper the retention is due to partitioning rather than to weak-complex formation. Their review paper [20] shows that there is research being done to better understand the role of the stationary phase in the retention process.

In the absence of a complete understanding of LC retention, empirical methodologies will continue to be used. In the following sections of this review article the empirical approach is pursued.

It is customary to denote the measured dissociation constant of an acid by K_a, even though the measured constant under experimental conditions is the apparent dissociation constant, K_{ap} as defined above. However, in the following discussion we will use K_a and pK_a to mean the measured apparent dissociation constant and the measured apparent pK_a respectively.

4.1. Column selection

The octadecyl silane (ODS) bonded-phase columns are used most commonly to separate ionogenic analytes and to determine their dissociation constants. Horváth et al. [9] and Van De Venne et al. [30] were among the first researchers to use the pH-dependent retention times of acidic compounds to determine their pK_a values by RP-HPLC. The compounds they studied were the relatively low molecular weight benzoic acid and its substituted derivatives, and ODS columns were quite suitable for this task. Shortly after these works were published, others reported the determination of pK_a values of certain drugs and other acid compounds by RP-HPLC using C-18 columns [31–34].

Miyake, Okumura and Terada [35] recognized that exact determination of the retention times of the neutral form and the ionic form of the compound was necessary to calculate pK_a values from chromatographic data. In their work they used a ODS column to improve the experimental methodology for the determination of the K_a's of benzoic acid, substituted benzoic acids, aniline, and substituted anilines. Szokoli et al. [17] used a ODS column in a study of the optimization of pH and solvent composition for the separation of organic acids, and in their article they reported pK_a values of organic acids calculated from their chromatographic measurements.

In their determination of dissociation constants of weak basic aroma compounds from natural products Bitteur and Rosset [36] used both an octadecylsilane column and a macroporous copolymer column, and data collected from either column gave the same pK_a value for a given compound. Data collected using an ODS C-18 column was used to calculate pK_a values of 2.45 and 3.85 for trimethylpyrazine and 2,4-dimethylthiazole, respectively, and with data from a copolymer column pK_a values for the two compounds were 2.46 and 3.85, respectively. Apparently there wasn't a problem using the ODS C-18 column below pH 3. Nomura et al. [37] found that the alkyl chain length of the ODS bonded-phase (C-8, C-10, C-18) did not have an effect on the separation of alkylphenols based on their dissociation constants.

When the molecular weight and the lipophilicity of the analyte increase, an ODS C-18 column may

retain the solute too strongly for convenient measurement of chromatographic parameters. In their first report of the determination of pK_a values of leukotriene B_4 and prostaglandin B_2 (which are essentially 20 carbon fatty acids), Hardcastle et al. [27] could not elute these compounds from an ODS C-18 column when the acetonitrile concentration of the mobile phase was less than 40%. Later, Hardcastle et al. [28] used a ODS C-8 column with better results in their study of the pK_a values of the leukotrienes.

Porous polystyrene copolymer columns have been used in the chromatographic determination of dissociation constants because they can be used over a wider pH range (pH 2–12) than can the octadecyl silane columns (pH 3–8). Pietrzyk et al. [12,13] pioneered the use of porous copolymers in their studies of the effect of solute ionization on chromatographic retention. These authors reported the use of various Amberlite XAD polymers to investigate the retention and separation of amino acids and peptides by HPLC over a wide pH range [14]. Palalikit and Block [38] point out that the adsorption characteristics of these nonionic copolymers should not change with the pH of the mobile phase. Ideally the mobile phase should be aqueous and the buffers used should be inorganic to minimize direct involvement with the stationary phase. However, it was found that elution times of unionized species were excessively long, and the eluted peaks were too broad to accurately measure retention times and thus, addition of an organic solvent to the mobile phase was necessary [38]. Recently, Miyake et al. [39] and Shibukawa et al. [40] reported the use of nonionic copolymer packings to determine the dissociation constants of aromatic acids, phenols, and aromatic nitrogen compounds.

For several years, Kirkland [41] has been championing the use of silica based columns with higher pH mobile phases when necessary, though most chromatographers do not use such columns outside the pH 3–8 range. Kirkland et al. (Ref. [41]) have done a great deal of research to demonstrate that silica based columns can be used at high pH's. Kirkland [41] suggests five actions that will increase the stability and lifetime of silica based columns used at high pH values. These actions are:

1. use supports based on sol-gel silica for more stability at high pH;
2. use densely packed, end capped long chain (C-18 or C-8) alkyl bonded phases;
3. use organic buffers instead of the typical phosphate and bicarbonate buffers;
4. use columns at temperatures below 40°C;
5. make sure that buffer ionic concentrations do not exceed 50 m*M*.

Kirkland [41] recommends using columns packed with highly purified acidic porous silica when ionizable analytes are involved.

Recently, Dolan [42] traced the history of silica based column packings in a brief but very readable article. With the newer type B silica columns, unwanted silanol interactions are reduced because these groups are shielded from interactions with the solute. The current status of analytical HPLC column technology is reviewed by Majors [43]. This author discussed the newer type B silica based and polymer column packings that have been developed in the past ten years. Majors [43] states that columns now have more efficiency, better stability, longer life, and can provide faster analyses and solve more separation problems.

4.2. Mobile phase composition

In early studies [9,12,13,38] of the determination of pK_a values by LC it was noted that the calculation depended on accurate experimental measurement of the capacity factors of the undissociated and fully dissociated form of the monoprotic weak acid. Likewise, for a weak base the capacity factor of the fully protonated and the ionized forms need to be accurately measured. Ideally, it would be desirable to be able to make these measurements in completely aqueous buffered mobile phases. However, most unionized organic acids (and fully protonated organic bases) would be retained too strongly under these conditions, and measurement of capacity factors would be difficult or not possible [38]. Thus, an organic solvent is added to the mobile phase to elute organic solutes more readily. However, addition of such an organic modifier will affect the ionization event of weak acids and bases [38,44].

Several organic solvents were studied by Palalikit

Table 4
Comparison of organic solvents used in buffer solution, pH 2.25

Solvent	% of solvent	ΔpH[c]	*k*	
			Benzoic acid	Sodium phenobarbital
Acetonitrile	5	0.10	39.00	VBP[b]
	10	0.13	14.50	VBP[b]
	15	0.13	8.60	13.60
Dioxane[a]	10	0.08	27.00	VBP[b]
	20	0.19	7.75	18.16
Methanol	20	0.14	no peak	no peak
	30	0.25	VBP[b]	VBP[b]
	40	0.30	21.00	36.90
Tetrahydrofuran	10	0.11	24.00	VBP[b]
	20	0.24	10.25	16.08

[a] Pressure increased approximately 300 psi.
[b] A very broad peak. [c] Increase or decrease in pH after addition of the acetonitrile to pH 2.25 buffer. (Reproduced from Palalikit and Block [38] with permission, copyright 1980, American Chemical Society).

and Block [38] in their determination of pK_a values of organic acids and bases. Table 4 shows the results of their work. It is noted that increasing the percentage of organic solvent decreases the k value as would be expected. Also, the increase in the percentage of organic solvent tends to increase the pH of the mobile phase, that is, an increase in the percent organic solvent affects the ionization events taking place in the buffered mobile phase. Fig. 3 shows that increasing the percentage of acetonitrile in the mobile phase decreases log *k* at a given pH of the mobile phase. The work of Hardcastle et al. [27,28] shows that the pK_a values of the leukotrienes increased with the percentage of acetonitrile in a nearly linear relationship. They used their results in this instance to do a linear extrapolation to obtain pK_a values of the leukotrienes in 100% water. While it is recognized that such linear extrapolations are to be accepted with some reservations, Hardcastle et al. [28] obtained a pK_a value of 4.22 for benzoic acid by this technique, and this value compares well with those obtained by other researchers using LC techniques.

Methanol is used widely as an organic modifier in the mobile phase when measuring parameters for the calculation of acid dissociation constants [17,30,35–37,44]. As is the case with any organic solvent, addition of methanol to the buffered aqueous phase changes the pH of the mobile phase [38] and thus, can affect the determination of pK_a values. Fig. 4 illustrates this with a plot of pK_a versus concentration of methanol in the mobile phase. Here it is seen that the relationship is not linear and Li et al.

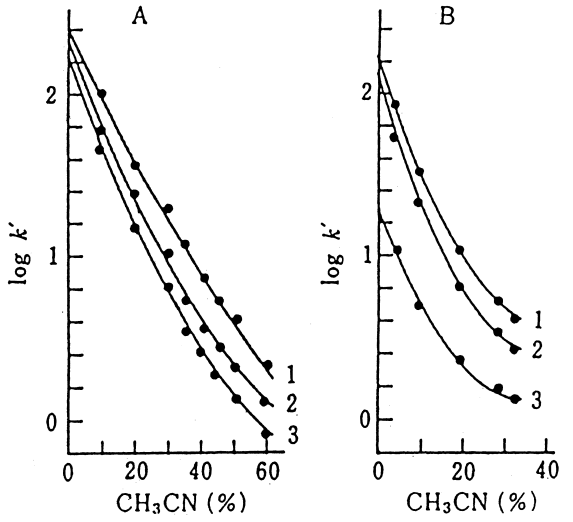

Fig. 3. Effect of CH_3CN Concentration in the mobile phase on log *k*; (A) Performed at pH 2.0. 1, benzene; 2. cyanobenzene; 3,4-methylphenol; (B) Performed at pH 11.0. 1,2-chloropyridine; 2. 2 methoxyaniline; 3, quinoline. The continuous lines in the figure represent change of *k* obtained by least squares calculation based on the quadratic relation between log *k* and the CH_3CN concentration. (Reproduced from Miyake et al. [39] with permission of the Pharmaceutical Society of Japan).

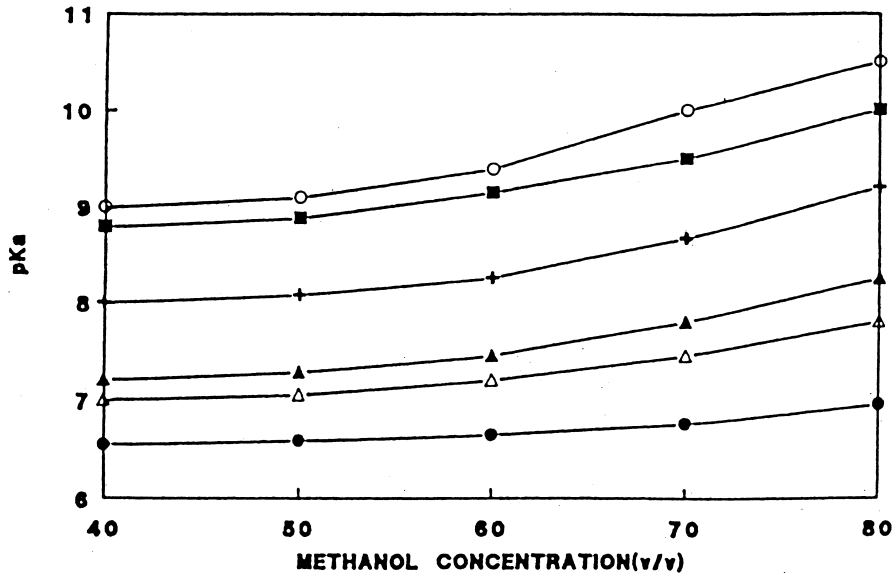

Fig. 4. Effect of the medium on the acidity constant. Temperature, 20°C; ionic strength, 0.02 *M* (phosphate buffer); (■) *o*-chlorophenol; (△) 2,6-dichlorophenol; (○) 6- chloroguaiacol; (●) 6-chlorovanllin, (+) 4,5- dichlorocatechol; and (▲) 3,4,5-trichlorocatechol. (Reproduced from Li et al. [44] with permission of Preston Publications. A Division of Preston Industries, Inc.).

[44] noted that the effect of methanol concentration is greater on weaker acids than on stronger acids. Miyake et al. [35] found that with acids the calculated pK_a values remained about the same up to 15% methanol, and then pK_a values increased at higher methanol concentrations. For basic analytes the pK_a values remained about the same up to 20% methanol, and then decreased at higher percentages of methanol. These authors point out that it is valuable to add up to 15% methanol to reduce retention times of more strongly retained solutes so that their retention times can be obtained conveniently and more accurately. This allows more exact measurement of the parameters needed for the calculation of dissociation constants. Szokoli et al. [17] found that methanol concentrations up to 30% did not affect the calculated pK_a value of the weak anthranilic acid.

4.3. Effect of buffer and ionic strength

From earlier discussion in this review, it is shown that ionic strength of the mobile phase will affect chromatographic retention of ionogenic solutes. Van De Venne et al. [30] found that ionic strength variation has little effect on the retention of undissociated solutes at low pH values, while it has a more pronounced effect on the dissociated acids at higher pH values. In general, increasing the ionic strength causes an increase of the retention factors of ionic species. Papp and Vigh [45] found, on the other hand, that the retention factor of aromatic amines decreased with increasing concentration of the buffer's cation. The magnitude of the retention- decreasing effect of inorganic buffer cations was in the following order: $H^+ < Na^+ < K^+$, irrespective of the type of organic amine solute. For example, in case of phosphate buffer, the authors found that the factor causing the change in the retention is not the overall phosphate concentration, but rather the concentration of sodium ion. The increase of the concentration of Na^+ has caused a decrease in the retention of all the amines examined. The authors concluded that the aromatic amines are retained by an ion-exchange mechanism rather than by hydrophobic effect. It is apparent, therefore, that the retention process is, in general, a complex phenomenon.

Otto and Wegscheider [46] found that at constant ionic strength, the retention of ionic species in reversed-phase chromatography is affected by the type of buffer used in the mobile phase. Changing

the buffer had little effect on the retention of the undissociated solutes, but it had a noticeable effect on the retention of the ionic solutes resulting from the dissociation of organic acids, amino acids, and dipeptides. The buffers used included phosphate, glycine, tartrate, acetate, and citrate. The effect of the buffer is attributed to masking the accessible silanols on the stationary phase. The authors concluded that the less polar the buffer, the less retained are the ionic species by the masked silanols groups.

González [47] also pointed out that it is necessary to take into consideration the non-ideal nature of the mobile-phase solvent mixture especially when there is no conservation in volume upon mixing solvents. González also suggests that the glass electrode used for measuring the pH of the buffered aqueous–organic solvent should be presoaked in the same solvent before measuring the pH.

5. Conclusion

In this review a representative sample of papers were considered from a large number of literature reports concerned with evaluating acid dissociation events in LC. Those interested in a great deal more information on this topic should consult the many references cited in the papers reviewed here. This review has attempted to present the theoretical basis and experimental methodologies that are needed to determine the pK_a values of a wide variety of pharmaceutical and biological compounds. The LC technique for determining acid dissociation constants is most useful when only small amounts of the analyte are available. Because numerous sample injections at various pH and mobile phase compositions are required to obtain the needed data, the LC technique is labor intensive.

6. List of abbreviations

H_nA	Polyprotic acid containing n acidic hydrogens
$H_{n-r}A^{r-}$	Anion with r negative charges
$K_{a,r}$	Dissociation equilibrium constant of the r^{th} dissociation step
$K(r)$	Dissociation constant of the formation of $H_{n-r}A^{r-}$ ($K(r) = \prod_{i=1}^{r} K_{a,i}$)
$a(H_{n-r}A^{r-})$	Activity of $H_{n-r}A^{r-}$
$\gamma(r)$	Activity coefficient of $H_{n-r}A^{r-}$
$\gamma(0)$	Activity coefficient of the neutral acid H_nA
$f(r) = \gamma(0)/\gamma(r)$	Activity coefficient ratio
[A]	Concentration of species A
$X(r)$	Mole fraction (relative concentration) of $H_{n-r}A^{r-}$
$K_{L,r}$	Equilibrium constant of the association between $H_{n-r}A^{r-}$ and the ligand L
k	Measured retention factor of the acid
k_r	Limiting retention factor of $H_{n-r}A^{r-}$
$K_e(r) = f(r) \cdot K(r)$	Effective dissociation constant of the formation of $H_{n-r}A^{r-}$ from H_nA
$K_{ap,i}$	Apparent dissociation constant of the i^{th} dissociation step of H_nA
ΔG	Free energy change
I	Ionic strength
ϵ	Dielectric constant

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